Phosphatidylinositol (PI), an important constituent of membranes, contains stearic acid as the major fatty acid at the sn-1 position. This fatty acid is thought to be incorporated into PI through fatty acid remodeling by sequential decylation and reacylation. However, the genes responsible for the reaction are unknown, and consequently, the physiological significance of the sn-1 fatty acid remains to be elucidated. Here, we identified acl-8, -9, and -10, which are closely related to each other, and ipla-1 as strong candidates for genes involved in fatty acid remodeling at the sn-1 position of PI. In both ipla-1 mutants and acl-8 acl-9 acl-10 triple mutants of Caenorhabditis elegans, the stearic acid content of PI is reduced, and asymmetric division of stem-like epithelial cells is defective. The defects in asymmetric division of these mutants are suppressed by a mutation of the same genes involved in intracellular retrograde transport, suggesting that ipla-1 and acl genes act in the same pathway. IPLA-1 and ACL-10 have phospholipase A and acyltransferase activity, respectively, both of which recognize the sn-1 position of PI as their substrate. We propose that the sn-1 fatty acid of PI is determined by ipla-1 and acl-8, -9, -10 and crucial for asymmetric divisions.

INTRODUCTION

Phosphatidylinositol (PI) is a versatile lipid that not only serves as a structural component of cellular membranes, but also plays important roles in signal transduction through distinct phosphorylated derivatives of the inositol head group (Di Paolo and De Camilli, 2006). The pathway for de novo synthesis of PI begins with the acylation of glycerol 3-phosphate (G3P) at the sn-1 position by G3P acyltransferase to form lysophosphatidic acid (lysoPA). LysoPA is further acylated at the sn-2 position by lysoPA acyltransferase to form phosphatic acid (PA), which serves as a general precursor for all phospholipids (Dircks and Sul, 1999; Wendel et al., 2009). PA is then converted to cytidine-diphosphodiacylglycerol (CDP-DAG), which combines with inositol to form PI. The newly synthesized PI possesses a mono- or di-unsaturated fatty acid at the sn-2 position (Akin and Shimojo, 1970; Holub and Kuksis, 1971a, 1972). In contrast, three-fourths or more of membrane PI obtained from mammalian tissues are constituted by the 1-stearoyl-2-arachidonoyl (18:0/20:4) species (Holub and Kuksis, 1972). In an RNA interference (RNAi)-based genetic screen, we recently identified mboa-7/LPIAT, a member of the membrane-bound O-acyltransferases (MBOAT) family, as an acyltransferase that selectively incorporates polyunsaturated fatty acids (PUFAs), such as AA and eicosapentaenoic acid (EPA), into the sn-2 position of PI (Lee et al., 2008). In this screen, we used the model organism Caenorhabditis elegans in which 1-stearoyl-2-eicosapentaenooyl (18:0/20:5) PI is the
major PI species (Supplementary Figure I; Lee et al., 2008). In C. elegans mbo-7/LPIAT mutants, EPA attached at the sn-2 position of PI was remarkably reduced and was replaced with other fatty acids such as oleic acid (18:1), indicating that fatty acid remodeling is crucial for determining the mature PI species.

On the other hand, many lines of evidence suggest that fatty acid remodeling also occurs at the sn-1 position of PI. In experiments with rabbit alveolar macrophage microsomes, newly synthesized PI from [14C]G3P contains palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2) at the sn-1 position, but very low levels of stearic acid (18:0), the predominant fatty acid esterified at the sn-1 position of tissue PI (Nakagawa et al., 1989). In addition, incubation of rat liver microsomes with dipalmitoyl (16:0/16:0) CDP-DAG and [14H]inositol results in the rapid synthesis of [14H]PI and [14H]lysoPI, the latter of which is subsequently reacylated with stearic acid (18:0) at the sn-1 position (Darnell et al., 1991a,b). This suggests that dipalmitoyl [14H]PI formed by the microsomes is rapidly hydrolyzed by phospholipase A2 (PLA2) to produce sn-2-acyl lysoPI, which is then reacylated with stearic acid by acyltransferase, which is present in the microsomes. In fact, a high level of stearoyl-CoA:sn-2-acyl lysoPI acyltransferase activity was detected in rat liver microsomes (Holub and Piekaraski, 1979; Darnell and Saltiel, 1991). These data strongly suggest that the fatty acid composition at the sn-1 position of PI is determined by the sequential actions of PLA2, and acyltransferase. However, the genes involved in this process have not been identified.

We previously showed that ipla-1, an intracellular PLA2 family member (Inoue and Aoki, 2006), acts as a regulator of asymmetric cell divisions in C. elegans (Kanamori et al., 2008). Loss of ipla-1 causes defects in asymmetric cell-fate determination and orientation of division of stem cell-like epithelial cells, called seam cells. We also found that a mutation of ibc-3 or mon-2, both of which are thought to be involved in endosome-to-Golgi retrograde transport (Lafourcade et al., 2004; Gillingham et al., 2006), suppresses the seam cell phenotypes of ipla-1 mutants. However, the role of ipla-1 in phospholipid fatty acid metabolism has not been determined. In this work, we analyzed the phospholipid fatty acid composition of ipla-1 mutants and found that the sn-1 fatty acid composition of PI was significantly altered in the mutants. Furthermore, we used evolutionarily conserved acyltransferases that are required for normal fatty acid composition of the sn-1 acyl moieties of PI.

**MATERIALS AND METHODS**

**Materials**

PI and lysoPI from bovine liver, lysoPC from egg yolk, dioleyl PA, dioleyl phosphatidylcholine (PC), dioleyl PE, 1-palmitoyl-2-oleoyl phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphatidylglycerol (PG) from egg yolk was purchased from Sigma-Aldrich (St. Louis, MO). Dipalmitoyl PI was purchased from Senday Research Laboratories (London, ON, Canada). [1-3C]palmitoyl-CoA, [1-3C]stearoyl-CoA, [1-3C]oleoyl-CoA, and [1-14C]arachidonyl-CoA were purchased from American Radiolabeled Chemicals (St. Louis, MO). Rhizopus delmer lipase was purchased from Seikagaku (Tokyo, Japan). PL2 from honey bee venom was purchased from Sigma-Aldrich.

**General Methods and Strains**

Worm cultures, genetic crosses, and other C. elegans methods were performed according to standard protocols (Brenner, 1974) except where otherwise indicated. The following mutations and transgenes were used: ipl1(ok143), acl-8 (acl-8tm10290), acl-8(tm10045), mon-2(xh22), ibc-3(tm323), act-2 (act-2p::gfp), pIR5, pIR6, pIR7 (Kage-Nakadai et al., 2010), ipl1(xh103), acl-8(xh103), dpy-7p::mCherry, xhEx3521[acl-8p::gfp; rol-6::GFP]), xhEx3523[acl-8p::gfp; dpy-7p::ipl-1::mCherry], xhEx3524[acl-8p::gfp; dpy-7p::ipl-1::mCherry], xhEx3529[acl-8p::gfp; dpy-7p::ipl-1::mCherry], tmEx1920[act-2p::ace-20::gfp; gcy-10p::DsRed1] (Kage-Nakadai et al., 2010), xhEx3514[scm::ac-10; Pges-1::dsRED6], xhEx3521[dpy-7p::mouse LYC]; Pges-1::dsRED1], xh3501[dpy-7p::acl-10; Pges-1::dsRED1]. The mutant alleles acl-8[stv-1] and ipl-1[ok143] were isolated in this study or provided by K. Gengyo-Ando (Kyushu University, Japan). Al mutations were backcrossed at least five times before further analysis.

**Cloning of C. elegans acl-10 and Mouse LYC**

acl-10 cDNA (GenBank accession number NM_001081071) was amplified by PCR from a C. elegans cDNA library using the primers, 5'-CAG AAT CTA GCA TGA TCA GTC CAT GTC-3' and 5'-AAA ATG GTA CCT TAT ATG GAA GAT GAT-3', and were cloned into pPDF49.78 (a gift from Dr. A. Fire, Stanford University) at the NheI and KpnI sites. Mouse LYC cDNA (GenBank accession number NM_001081071) was amplified from a cDNA library derived from mouse heart using the primers, 5'-CCC GGC TAC CAA ATG GTC TTA CTT CT-3' and 5'-GGG CAT CAG TCA GGT GAT CAT TTT TT-3'. Some of the experiments used in this work were obtained from C. elegans strains cultured in the Kage-Nakadai Genetics Center (University of Minnesota, Minneapolis, MN). All mutations and backcrossed strains were analyzed at least five times before further analysis.

**Constructs and Transgenic Worms**

For each construct, more than three independent transgenic lines were analyzed. pIR1 (acl-10p::gfp), pIR2 (acl-10p::acl-10::gfp), pIR3 (dpy-7p::acl-10::mCherry), pIR4 (acl-10p::acl-10::mCherry) and pIR7 (dpy-7p::mouse LYC) were prepared as follows. pIR1 (acl-10p::gfp): 0.6-kb gene fragment immediately upstream the ATG initiation codon of acl-10 was PCR amplified using the primers 5'--AAG TGG TCT GGC AAG GAT GAT GAT-3' and 5'--GAG ATG GTC TTA CTT CT-3', and 5'--ATG TGC GGA AGA GAA CTT GT-3', and cloned into pPDF49.78 (NLS-; a derivative of pPDF49.67, a gift from A. Fire, Stanford University School of Medicine) at the BamHI and HindIII sites. pIR4 (acl-10p::acl-10::mCherry) and pIR7 (dpy-7p::mouse LYC) were prepared as follows. pIR1 (acl-10p::gfp): 0.6-kb gene fragment immediately upstream the ATG initiation codon of acl-10 was PCR amplified using the primers 5'--AAG TGG TCT GGC AAG GAT GAT GAT-3' and 5'--GAG ATG GTC TTA CTT CT-3', and 5'--ATG TGC GGA AGA GAA CTT GT-3', and cloned into pPDF49.67 (NLS- at the BamHI and HindIII sites. pIR3 (dpy-7p::acl-10:: acl-10 cDNA was subcloned under a dpy-7 promoter in a pTK030 (Kanamori et al., 2008) at the Smal and NotI sites. pIR4 (acl-10p:: acl-10 cDNA was subcloned under a dpy-7 promoter in a pTK020 (Kanamori et al., 2008) at the Smal and NotI sites. pIR5, pIR6 (acl-10p::acl-10::mCherry), acl-10p::acl-10::gfp were PCR amplified from pTK030 (Kanamori et al., 2008) and were isolated in this study by TMP (trimethylp-...
PLA Assay
PLA assay using recombinant IPIA-1 was performed as described previously (Morikawa et al., 2007), except that human embryonic kidney (HEK) 293 cells were used instead of HeLa cells. Purified recombinant protein was incubated with liposomes containing each phospholipid (dioleoyl PA, dioleoyl PC, dioleoyl PE, 1-palmitoyl-2-oleoyl PS, and dipalmitoyl PS) at 37°C for 5 h. The released free fatty acid level was measured with the ACS-ACOD method (NEFA C-Test kit 279–75401, Wako Chemical, Osaka, Japan).

Acyltransferase Assay
sn-2-acyl-1-lysophospholipids (sn-2-acyl lysophospholipids) were prepared as described previously (Lee et al., 2008) using dioleoyl PC, dioleoyl PE, sn-1-palmitoyl-2-oleoyl PS, bovine liver PI, and egg yolk PG. Each sn-2-acyl lysophospholipid was immediately used for acyltransferase assay. Because sn-2-acyl lysophospholipid is known to easily isomerize to sn-1-acyl-2-lysophospholipid (briefly, a 1-acyl lysophospholipid), it is possible that 4-acyl donor is incorporated into sn-1-acyl lysophospholipid and sn-2-acyl lysophospholipid (Supplementary Figure 2A). Thus, an accurate measure of sn-2-acyl lysophospholipid acyltransferase activity could only be obtained by determining the position that had been acylated. The acyltransferase reaction mixtures contained 80 μM lysophospholipid, 12.5 μM [14C]acyl-CoA (55 mCi/mmol), and 50 μg of microsomal protein of C. elegans in a total volume of 0.8-ml assay buffer (0.15 M KCl, 0.25 M sucrose, 50 mM potassium phosphate buffer [pH 6.8]). After incubation at 37°C for 5 min, reactions were stopped by adding 2 ml of methanol. Total lipid was extracted by the method of Bligh and Dyer, and separated by one-dimensional TLC on silica gel 60 plates (Merck) in chloroform/methanol/acetic acid/water (50:30:8:4, vol/vol). To check the positional specificity, the radiolabeled product was reextracted from the TLC plates and treated with bee venom PLÅ (Supplementary Figure 2). The distribution of radioactivity between the resultant sn-1-acyl lysophospholipid and free fatty acids was assessed after TLC in chloroform/methanol/acetetic acid/water (50:30:8:4, vol/vol). Acyl-CoA-sn-2-acyl lysophospholipid acyltransferase activity was determined by the radioactivity of sn-1-acyl lysophospholipid (Supplementary Figure 2, B and C). Acyl-CoA-sn-1-acyl lysophospholipid acyltransferase assay was performed essentially as described previously (Lee et al., 2008), except that 50 μg of microsomal protein and 80 μM lysophospholipid from bovine liver were used for the acyltransferase assay. Acyltransferase activity of the HEK 293 cell microsomes was measured similarly, except that the incubation temperature was 37°C.

Cell Culture and Transfection
HEK 293 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 1-glutamine (2 mM). Transfection of the plasmid DNA into cells was performed using LipofectAMINE 2000 (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

Microscopy and Phenotypic Analysis
Animals were mounted on a 5% agar pad on a glass slide and immobilized in 0.02 M azide. Fluorescence images were obtained using an Axios Imager M (Carl Zeiss Microimaging, Thornwood, NY). Confocal images were obtained using a Zeiss LSM510META confocal microscope system (Carl Zeiss Microimaging, Thornwood, NY). The orientation of the cell and divisions of seam cells was analyzed as previously described (Kanamori et al., 2008). The angle between the line along the A-P axis was calculated. The angle outside the range of ±10° was defined as abnormal.

Western Blot Analysis
Mix-stage populations of worms were collected and sonicated in SET buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose) with protease inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml apronitin, and 1 mM PMSF). After sonication, the lysates were centrifuged at 10,000 × g for 10 min at 4°C. The resulting supernatants were further centrifuged at 100,000 × g for 60 min at 4°C. The supernatant (soluble fraction) and the supernatant (mitochondrial fraction) (500 and 1000, respectively) were subjected to SDS-PAGE and immunoblotting. The antibodies used were antibodies to the immuneblots and their dilutions were anti-IPIA-1 polyclonal antibody (Kanamori et al., 2008), 1:20; anti-mCherry mAb (632543, Clontech), 1:1000; anti-a-tubulin mAb (DM1A, Sigma) 1:5000; and anti-green fluorescent protein (GFP) mAb (JL-8, Clontech), 1:1000.

RESULTS
ipla-1 Mutation Causes Reduced Stearic Acid Content of PI
PLA1 is an enzyme that hydrolyzes fatty acids attached at the sn-1 position of phospholipids. The C. elegans genome contains one intracelluar PLA1 family member named ipla-1 (Kanamori et al., 2008). To determine the role of ipla-1 in phospholipid metabolism, we first analyzed the fatty acid composition of phospholipids in wild-type and ipla-1 mutants by GC (Figure 1, A–D). GC analysis of PC, PE, PS, and PI revealed that the ipla-1 mutation significantly affected the fatty acid composition of PI, but not the fatty acid compositions of PC, PE, or PS. In ipla-1 mutants, the amount of stearic acid (18:0), which is the major fatty acid in the sn-1 position of PI, was reduced to 6.0% of total PI fatty acids compared with 25.1% in wild-type animals. Conversely, the amount of vaccenic acid (18:1n-7) in PI increased to 25.6% of total PI fatty acids compared with 16.0% in wild-type animals. The amount of EPA (20:5), the predominant fatty acid at the sn-2 position of PI in C. elegans (Supplementary Figure 1; Lee et al., 2008) was not affected. Consistent with this observation, LC/ESI-MS analysis revealed that the amount of 180/20:5-PI was reduced and instead, 181/20:5-PI was increased in ipla-1 mutants (Figure 2). These data indicate that ipla-1 is required for the incorporation of 18:0 into the sn-1 position of PI.

Fatty Acid Composition of PI in acl-8 acl-9 acl-10 Triple Mutants Is Similar to That in ipla-1 Mutants
Decaylating-reacylation of phospholipids by PLA and acyltransferase is assumed to be important for acquiring the appropriate fatty acid composition of membrane phospholipids (Lands, 1958; Waku and Nakazawa, 1972; Lands, 2000). So far, several acyltransferases that incorporate fatty acids into the sn-2 position of lysophospholipids have been identified and are classified into two gene families, the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) family and the MBOAT family (Shindou and Shimizu, 2009). We have isolated all mutants of AGPAT family members in C. elegans (acl-1;14; Supplementary Table 1) and found that the triple mutants of acl-8, -9, and -10 (Supplementary Figure 4), which are closely related to each other (Supplementary Figure 4), exhibited vulval defects similar to those of ipla-1 mutants (see below). This observation led us to examine the fatty acid composition of phospholipids in acl-8 acl-9 acl-10 triple mutants [acl-8 acl-9 (tm2290) acl-10 (tm1045)].

In acl-8 acl-9 acl-10 mutants, the amount of stearic acid (18:0) in PI was reduced (25.1% in wild-type vs. 10.0% in acl-8 acl-9 acl-10 mutants), whereas the amount of vaccenic acid (18:1n-7) in PI increased (16.0% in wild-type vs. 27.5% in acl-8 acl-9 acl-10 mutants) in a similar manner to ipla-1 mutants (Figure 1A). The amount of EPA (20:5) in PI was unaltered. LC/ESI-MS analysis showed that 180/20:5-PI was replaced by 181/20:5-PI in acl-8 acl-9 acl-10 mutants like it was in ipla-1 mutants. No drastic changes were observed in the fatty acid compositions of other phospholipids such as PC, PE, and PS (Figure 1, B–D). These data indicate that acl-8, -9, and -10 are also involved in the incorporation of 18:0 into the sn-1 position of PI.

Phenotypes of acl-8 acl-9 acl-10 Triple Mutants Are Similar to Those of ipla-1 Mutants
We previously demonstrated that ipla-1 mutants exhibited defects in vulval morphology (Supplementary Figure 5, A and B) and cell division of lateral epithelial cells, termed seam cells (Figure 3 and Table 1; Kanamori et al., 2008). In the course of constructing the acl-8 acl-9 acl-10 triple mutants used above, we noticed that acl-8 acl-9 acl-10 mutants, like ipla-1 mutants, showed vulval defects, including protruding and bursting vulvae (Supplementary Figure 5, A–C). To address whether acl-8 acl-9 acl-10 mutants also show abnormal cell division of seam cells, we analyzed the seam cell divisions of acl-8 acl-9 acl-10 mutants using a seam cell
marker, scm::gfp. During the larval stages, seam cells divide asymmetrically in a stem cell-like manner, producing an anterior daughter cell that fuses with a major epithelial syncytium (hyp7) and loses the expression of scm::gfp, and a posterior daughter cell that assumes the seam cell fate again and continues to express scm::gfp (Figure 3A). Each seam cell division is oriented parallel to the anterior-posterior axis (A-P axis). Because orientation of seam cell division is randomized relative to the A-P axis in ipla-1 mutants (Kanamori et al., 2008), we first analyzed the orientation of seam cell division. In wild-type worms, all seam cells divided parallel to the A-P axis as judged by scm::gfp, which is expressed in the nuclei of both daughter cells just after the division (Figure 3B and Table 1). On the other hand, ipla-1 mutants exhibited aberrant orientation of seam cell divisions as reported previously (Figure 3C and Table 1; Kanamori et al., 2008). These observations indicate that acl-8 acl-9 acl-10 mutants, like ipla-1 mutants, are defective in orientation and cell-fate determination of seam cell divisions.

In C. elegans, the Wnt/β-catenin asymmetry pathway determines the cell fate of most asymmetric divisions (Mizumoto and Sawa, 2007b). In asymmetric division of seam cells, Wnt is expressed posterior to the mother cell before dividing, and this polarity information is converted to anterior cortical localization of WRM-1::GFP in mother seam cells at the L4 stage in acl-8 acl-9 acl-10 mutants. In wild-type seam cells, punctate fluorescence was clearly visible near the cell membrane in the anterior half of the cells (Figure 4A). On the other hand, ipla-1 mutants exhibited aberrant orientation of seam cell divisions as reported previously (Figure 3B and Table 1; Kanamori et al., 2008). acl-8 acl-9 acl-10 triple mutations also caused misorientation of seam cell divisions similar to that observed in ipla-1 mutants (i.e., the seam cells divided obliquely, and sometimes at right angles to the A-P axis; Figure 3D and Table 1). Next, we investigated the cell lineage pattern of seam cells at the L4 stage in acl-8 acl-9 acl-10 mutants. As mentioned above, in wild-type worms, all posterior daughter cells maintained expression of scm::gfp and assumed the seam cell fate again (Figure 3E). In contrast, the seam cell division became symmetric or was reversed (the fates of daughters were the opposite of what they are in the wild type) in ipla-1 mutants (62%, n = 53; Figure 3F; Kanamori et al., 2008). Similarly, the asymmetry of the divisions was often disrupted in acl-8 acl-9 acl-10 mutants (67%, n = 25; Figure 3G). These observations indicate that acl-8 acl-9 acl-10 mutants, like ipla-1 mutants, are defective in orientation and cell-fate determination of seam cell divisions.

Figure 1. Fatty acid composition of PI (A), PC (B), PE (C), and PS (D) from wild-type, ipla-1 mutants and acl-8 acl-9 acl-10 triple mutants. GC analysis was used to measure the individual fatty acid species. cyclo, total cyclopropane fatty acids (17: Δ, 19: Δ); branched, branched fatty acids (15:0 iso, 15:0 ante, 16:0 iso, 17:0 iso, 17:0 ante); others, unidentified fatty acids. Bars, mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
and occasionally, we observed cytoplasmic puncta of WRM-1::GFP similar to those in ipla-1 mutants (Figure 4, B and C). These results indicate that both ipla-1 and acl-8, -9, -10 are required for the formation and/or maintenance of cortical asymmetry of WRM-1 before the divisions of the seam cells.

**Suppressor Mutations of ipla-1 Suppress the Seam Cell Defects of acl-8 acl-9 acl-10 Triple Mutants**

In a previous genetic screen, we isolated two alleles, tbc-3(xh23) and mon-2(xh22), as suppressors of the seam cell defects of ipla-1 mutants (Kanamori et al., 2008). mon-2 encodes a homologue of an ARF GEF-like protein and tbc-3 encodes a homologue of a Rab GAP, both of which have been reported to regulate endosome-to-Golgi retrograde transport (Lafourcade et al., 2004; Gillingham et al., 2006). We also found that a seam cell-specific RNAi for the retromer complex, which is also known to control intracellular retrograde transport (Seaman, 2005), suppresses the seam cell phenotypes of ipla-1 mutants, suggesting that reduction of endosome-to-Golgi retrograde transport in seam cells rescues the seam cell defects of ipla-1 mutants (Kanamori et al., 2008). In this study, we found that mutation of tbc-3 did not appreciably change the PI molecular species in ipla-1 mutants (Supplementary Figure 6), suggesting that the mutation suppresses seam cell phenotypes by abnormal membrane traffic downstream of altered PI molecular species.

To address whether the seam cell defects of acl-8 acl-9 acl-10 mutants are also mediated by retrograde transport, we crossed acl-8 acl-9 acl-10 mutants with tbc-3(xh23) or mon-2(xh22) mutants and analyzed seam cell divisions. The orientation of seam cell divisions was restored in both acl-8 acl-9 acl-10; tbc-3 and acl-8 acl-9 acl-10; mon-2 mutants. The percent of the seam cells in which the angle between the A-P axis and the direction of cell division was more than 10° was 6% in the acl-8 acl-9 acl-10; tbc-3 and acl-8 acl-9 acl-10; mon-2 mutants, whereas it was 41% in the acl-8 acl-9 acl-10 mutants (Figure 5, A and B; Table 1). The abnormal localization of WRM-1::GFP observed in the mother seam cells of acl-8 acl-9 acl-10 mutants was also rescued by a mutation of tbc-3(xh23) or mon-2(xh22) (Figure 5, C and D). These results suggest that the seam cell defects of ipla-1 mutants and acl-8 acl-9 acl-10 mutants occur through the same pathway, which is mediated by retrograde transport.

**Figure 2.** MS analysis of PI molecular species. Negative ionization LC/ESI-MS spectra of PI molecular species of wild-type (top), ipla-1 mutants (middle), and acl-8 acl-9 acl-10 triple mutants (bottom). Assigning specific molecular species to m/z values was based on their calculated theoretical monoisotopic masses and verified by MS/MS. Lower case a refers to alkyl ether linkage.
Recombinant IPLA-1 Hydrolizes a Fatty Acid Attached to PI

Mammals have at least three members of the intracellular PLA family, namely PA-PLA1, KIAA0725, and p125, all of which show significant homology to an ipla-1 gene product (IPLA-1; Higgs et al., 1998; Nakajima et al., 2002; Kanamori et al., 2008). PA-PLA1 and KIAA0725 have been reported to hydrolyze the sn-1 fatty acids attached to PI in vitro assays (Higgs and Glomset, 1996; Morikawa et al., 2007). To determine whether C. elegans IPLA-1 also has hydrolytic activity toward PI, we prepared recombinant IPLA-1 by expressing it in HEK cells (Figure 6A, see Materials and Methods). As shown in Figure 6B, the purified recombinant IPLA-1 showed hydrolytic activity toward PI. PS and PC were also hydrolyzed by IPLA-1, but PE or PA did not serve as a substrate (Figure 6B).

ACL-10 Possesses Aciyltransferase Activity toward the sn-1 Position of PI

acl-10 single mutants showed defects in vulval morphology and seam cell divisions comparable to those of acl-8 acl-9 acl-10 triple mutants, whereas acl-8 acl-9 double mutants exhibited no abnormalities in vulval morphology or seam cell divisions (Table 1 and Supplementary Figure 5, C–E and I–K), indicating that acl-10 predominantly contributes to vulval morphology and seam cell divisions. We therefore focused on acl-10 and examined whether an acl-10 gene product (ACL-10) has LPIAT activity toward the sn-1 position of PI. The membrane fraction of ACL-10-expressing transgenic worms (xhIs3501[dpy-7p::acl-10]; see Materials and Methods) showed increased sn-2-acyl LPIAT activity with stearoyl-CoA as an acyl donor (Figure 6C). Aciyltransferase activities against other sn-2-acyl lysophospholipids, such as lysyPC, lysyPE, lysyPS, and lysyPG, did not increase significantly (Figure 6C), indicating that ACL-10 prefers sn-2-acyl lysyPI as an acyl acceptor. Increased sn-2-acyl LPIAT activity was also observed when we used palmitoyl-CoA (16:0-CoA) and oleoyl-CoA (18:1n-9-CoA), but not arachidonoyl-CoA (20:4n-6-CoA), as acyl donors (data not shown). These data indicate that ACL-10 has acyltransferase activity toward the sn-1 position of PI with a preference for saturated and mono-unsaturated fatty acids. In the membrane fraction of acl-8 acl-9 acl-10 mutants, sn-2-acyl LPIAT activity was significantly reduced when we used stearoyl-CoA (18:0-CoA) as the acyl donor (Supplementary Figure 7). However, it was not reduced when we used arachidonoyl-CoA (20:4n-6-CoA), which is the preferred acyl donor of MBOA-7/LPIAT. This result indicates that ACL-8, -9, and -10 contribute to sn-2-acyl LPIAT activity with stearoyl-CoA, but not with arachidonoyl-CoA, in C. elegans. The observation that appreciable activity was still detected in the membrane fraction of acl-8 acl-9 acl-10 mutants suggests the existence of other sn-2-acyl LPIAT in the worms.

IPLA-1 and ACL-10 Are Expressed in the Endoplasmic Reticulum of Epithelial Cells

We also examined the expression pattern of acl-10 by using a transcriotional GFP fusion gene. Strong acl-10p::GFP expression was observed in seam cells throughout development (Figure 7A). Expression of GFP was also observed in other epithelial cells, such as vulval epithelial cells and the major epithelial syncytium hyp7, and in several head

Table 1. Orientation of seam cell division

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of cell divisions with abnormal orientation</th>
<th>n</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td>ipla-1</td>
<td>52.4</td>
<td>107</td>
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<td>104</td>
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<td>107</td>
</tr>
<tr>
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<td>5.7</td>
<td>147</td>
</tr>
<tr>
<td>acl-8 acl-9 acl-10: mon-2</td>
<td>5.5</td>
<td>109</td>
</tr>
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Figure 3. ipla-1 mutants and acl-8 acl-9 acl-10 triple mutants are defective in the orientation and asymmetric cell-fate determination of seam cell divisions. Anterior is toward the left. (A, top) Schematic arrangement of seam cells on each side in an early L1 larva. (A, bottom) The postembryonic division pattern of V seam cells. The expression pattern of scm::gfp, which is specifically expressed in the nuclei of seam cells, is indicated by green lines. The box marks the lineages analyzed in this study: V5.pppp lineage (a), V6.papp lineage (b), and V6.pppp lineage (c). Gray circles represent anterior daughters that fuse with the epithelial syncytium, hyp7, and green squares denote seam cells. Red and blue lines indicate the developmental stages corresponding to those of B–D and E–G, respectively. (B–D) Fluorescent images of scm::gfp just after the divisions at the L4 stage. The shapes of the worms are indicated by dotted lines. Three pairs of daughter cells are shown with brackets. (B) Wild-type. All seam cells divide parallel to the A-P axis. (C and D) Representative scm::gfp images of ipla-1 mutants (C) and acl-8 acl-9 acl-10 triple mutants (D). In ipla-1 mutants and acl-8 acl-9 acl-10 triple mutants, the seam cell division is randomly oriented relative to the A-P axis (C and D; *). (E–G) Seam cells at the late L4 stage visualized by scm::gfp. Merged fluorescence and differential interference contrast images are shown. The letters (a), (b), and (c) correspond to those of A. (E) Wild-type. The posterior daughter cells adopt the seam cell fate in all the three lineages. (F and G) Representative scm::gfp images of ipla-1 mutants (F) and acl-8 acl-9 acl-10 triple mutants (G). The asymmetry of the divisions is often disrupted in ipla-1 mutants and acl-8 acl-9 acl-10 triple mutants. Scale bars, 20 μm.
neurons including AIY interneurons (Figure 7, B and C). To analyze the intracellular localizations of IPLA-1 and ACL-10, we generated a transgenic strain expressing IPLA-1::mCherry and ACL-10::GFP under the control of the epidermal-specific dpy-7 promoter and acl-10 own promoter, respectively. IPLA-1::mCherry and ACL-10::GFP rescued the phenotypes of ipla-1 mutants and acl-8 acl-9 acl-10 mutants, respectively (Supplementary Figure 5, F and G), indicating that these fusion proteins are functional. As shown in Figure 7D, IPLA-1::mCherry and ACL-10::GFP were distributed in an endoplasmic reticulum (ER)-like reticular pattern throughout the cytoplasm and were partially colocalized. We also found that both IPLA-1 and ACL-10 partially colocalized with an ER marker, ACS-20::EGFP (Supplementary Figure 8; Kage-Nakadai et al., 2010). Furthermore, an immunoblot analysis revealed that both IPLA-1 and ACL-10 were present in the soluble fraction (Figure 7E). IPLA-1 was also present in the soluble fraction (Figure 7E), as was its mammalian homologue, KIAA0725 (Nakajima et al., 2002; Morikawa et al., 2009). These data indicate that ACL-10 and a portion of IPLA-1 are localized at the ER membrane in epithelial cells.

**DISCUSSION**

**Identification of ipla-1 and acl-8, -9, -10 as Strong Candidates for Enzymes Involved in Fatty Acid Remodeling at the sn-1 Position of PI**

PI is a membrane phospholipid that has a unique fatty acid composition in that 1-stearoyl-2-arachidonoyl species is predominant in mammals (Holub and Kuksis, 1971a; Baker and Thompson, 1972). *C. elegans*, in which EPA is a major PUFA in membrane phospholipids and AA is a minor component, possesses 1-stearoyl-2-eicosapentaenoyl PI as the major molecular species (Lee et al., 2008). These molecular species are thought to be formed by a fatty acid remodeling reaction after the de novo synthesis of PI (Holub and Kuksis, 1971a; Nakagawa et al., 1989; Darnell et al., 1991a,b). The remodeling reaction involves the hydrolysis of a fatty acyl ester bond at the sn-1 or -2 position of the newly synthesized phospholipids and subsequent incorporation of the appropriate fatty acid into the position. We recently identified the acyltransferase, named mboa-7/LPIAT, which preferentially incorporates AA and EPA into the sn-2 position of PI (Lee et al., 2008). In the present study, we identified PLA1 (ipla-1) and acyltransferases (acl-8, -9, -10) that are involved in the incorporation of stearic acid into the sn-1 position of PI. We demonstrated that 1) stearic acid (18:0) attached at the sn-1 position of PI is replaced with vaccenic acid (18:1n-7) in both *ipla-1* mutants and *acl-8 acl-9 acl-10* mutants, 2) *ipla-1* mutants and *acl-8 acl-9 acl-10* mutants show similar phenotypes (i.e., they exhibit defects in orientation of seam cell divisions, cell-fate determination of seam cells, and cortical localization of β-catenin in mother seam cells), and 3) these phenotypes are suppressed by a same mutation (*tbc-3(xh23) or *mon-2(xh22)*) in both *ipla-1* mutants and *acl-8 acl-9 acl-10* mutants. Together, these data strongly suggest that *ipla-1* (PLA1) and *acl-8, -9, -10* (acyltransferases) function in the same pathway. We also showed that *ipla-1* mutants, *acl-8 acl-9 acl-10* mutants, and *ipla-1; acl-8 acl-9 acl-10* quadruple mutants have seam cell defects with similar penetrance (Table 1) and have similar fatty acid compositions of PI (Supplementary Figure 9), indicating no synergism between the *ipla-1* and *acl-8 acl-9 acl-10* mutations. These data further support the idea that *ipla-1* and *acl-8, -9, -10* function in the same pathway. In in vitro analyses, IPLA-1 was capable of hydrolyzing the fatty acyl moiety of PI, and ACL-10 transferred stearic acid to the sn-1 position of PI. From these observations, we propose that *ipla-1* functions as a PLA1 and that *acl-8, -9, -10* function as acyltransferases in the
fatty acid remodeling of the sn-1 position of PI (Supplementary Figure 10).

Calcium-independent PLA2 (iPLA2) has been assumed to play a role in fatty acid remodeling of the sn-2 position of phospholipids (Balsinde et al., 1997). Recent knockout studies, however, revealed that lack of iPLA2/H9252 causes no significant change in the fatty acid composition of membrane phospholipids (Bao et al., 2007). Knockout mice of iPLA2/H9253, which hydrolyze the sn-2 ester bond of PC, show marked differences in fatty acid composition of cardiolipin (Mancuso et al., 2007), although the mechanism by which the fatty acid composition of cardiolipin is changed is unclear. As for PLA1, three members of the intracellular PLA1 family (PA-PLA1, KIAA0725, and p125) have been identified and analyzed (Inoue and Aoki, 2006; Morikawa et al., 2009), although the contribution of these PLA1s to fatty acid composition of membrane phospholipids is unknown. The present results show that ipla-1 mutants, in which PI-recog-
nizing PLA1 is disrupted, have altered fatty acid composition of PI and seam cell defects similar to those observed in the acl-8 acl-9 acl-10 mutants, which lack acyltransferases for transferring the fatty acid at the sn-1 position of PI. To our knowledge, ipla-1 is the first identified phospholipase involved in the fatty acid remodeling at the sn-1 position of membrane phospholipids. In an in vitro analysis, IPLA-1 also showed hydrolytic activity toward PS and PC in addition to PI (Figure 6B), although the ipla-1 mutation did not appreciably affect the fatty acid composition of PS and PC in vivo (Figure 1). It is possible that IPLA-1 cleaves the sn-1 fatty acyl bond of PS and PC under our in vitro condition,

Figure 6. Enzymatic assay of IPLA-1 and ACL-10. (A) Purification of IPLA-1 expressed in HEK 293 cells was verified by SDS-PAGE followed by Coomassie Brilliant Blue staining. Left, lysates of HEK 293 cells transfected with ipla-1 cDNA in the pFLAG-CMV2 vector (Kanamori et al., 2008). Right, FLAG-IPLA-1 purified with anti-FLAG M2 affinity gel and eluted with buffer containing the FLAG peptide. (B) Substrate specificity of IPLA-1. The following phospholipids were used for substrates; dioleoyl PA, dioleoyl PC, dioleoyl PE, 1-palmitoyl-2-oleoyl PS, and dipalmitoyl PI. For details of the assay procedure, see Materials and Methods. (C) Substrate specificity of ACL-10. Acyltransferase activities of wild-type (ⅰ) or ACL-10–expressing transgenic worms (ⅱ) were measured using [14C]stearyl-CoA as an acyl donor and the indicated sn-2-acyl lysophospholipids as acyl acceptors. Bars, mean ± SEM of at least three independent experiments. **p < 0.01.

Figure 7. Expression of ipla-1 and acl-10. (A–C) Confocal images of transgenic worms expressing acl-10p::GFP. Seam cells (A–C: arrowheads), epithelial syncytium hyp7 (A: bracket), vulval epithelium (B: arrows), and several neurons including AIYL/R (C: arrowheads) in the head region at the adult stage. Scale bars, 40 μm. (D) Confocal images of epithelial cells of a transgenic L4 worm expressing IPLA-1::mCherry and ACL-10::GFP. Bar, 20 μm. (E) Subcellular fractionation of IPLA-1 and ACL-10. Lysate of transgenic worms expressing ACL-10::mCherry and ACS-20::EGFP (acs-20;acs-22;xhEx3529[acl-10p::acl-10::mCherry]; tmEx1920[acs-20p::acs-20::egfp]) were subjected to S100 (cytosol)/P100 (membrane) fractionation and processed for immunoblotting with anti-IPLA, anti-mCherry (ACL-10), anti-α-tubulin (cytoplasmic marker), and anti-GFP (ER marker ACS-20) antibodies. acs-20 encodes a very long chain fatty acid acyl-CoA synthetase that is known to be localized in ER membranes. acs-22 is a homologous gene of acs-20; acs-20p;acs-20::egfp transgene fully rescues the phenotypes of acs-20; acs-22 double mutants (Kage-Nakadai et al., 2010).
but not in vivo. Alternatively, lysoPS and lysoPC produced by IPLA-1 might be reacylated back to their original states by certain acyltransferases in vivo.

According to the model shown in Supplementary Figure 10, mutations of acl-8, -9, -10 are expected to accumulate lysoPI, but not to accumulate 18:1/20:5-PI. However, in acl-8 acl-9 acl-10 mutants, the amount of 18:1/20:5-PI increased (Figures 1 and 2), and lysoPI was not detected by our measuring system (data not shown). This may be because acyltransferases other than ACL-8, -9, -10 transfer 18:1, the principal fatty acid in C. elegans (Hutzell and Krusberg, 1982), to the sn-1 position of PI in acl-8 acl-9 acl-10 mutants. It is also possible that IPLA-1 and ACL-8, -9, -10 activities are coupled so that IPLA-1 is inactive when ACL-8, -9, -10 are lost.

As mentioned above, acl-10 single mutants showed seam cell defects comparable to those of acl-8 acl-9 acl-10 triple mutants, whereas acl-8 acl-9 double mutants exhibited no abnormalities (Table 1), indicating that acl-10 predominantly contributes to seam cell divisions. In acl-10 single mutants, the amount of 18:0/20:5-PI was reduced and the amount of 18:1/20:5-PI was increased compared with the amounts in wild-type worms. However, the changes were less than those observed in acl-8 acl-9 acl-10 mutants (Supplementary Figure 11A). As shown in Figure 7A, acl-10 is strongly expressed in seam cells, but is not expressed in intestinal cells or muscle cells, which form large organs in C. elegans. We also found that acl-10 mRNA obtained from the whole C. elegans body was less than that of acl-8 or -9 as judged by quantitative real-time PCR (Supplementary Figure 11B). Therefore, the local expression of acl-10 appears to be the cause of the weak change of PI molecular species in acl-10 single mutants.

Several laboratories, including ours, have identified acyltransferases that incorporate fatty acids into the sn-2 position of lysophospholipids (Shindou and Shimizu, 2009). However, the acyltransferases transferring fatty acids at the sn-1 position of lysophospholipids are largely unknown. Very recently, another acyltransferase, Psi1p, was shown to catalyze acyl transfer to the sn-1 position of PI in yeast (Le Guedard et al., 2009). In psi1 Δ mutants, the content of stearic acid at the sn-1 position of PI is reduced, but the physiological function of Psi1p has not been elucidated. We show here that the C. elegans acyltransferase toward the sn-1 position of PI is required for asymmetric cell division, the fundamental mechanism by which multicellular organisms generate cell diversity.

Asymmetric Cell Division and Fatty Acid Remodeling of PI

The present results demonstrate that both ipla-1 mutants and acl-8 acl-9 acl-10 mutants have defects in asymmetric cell divisions of stem-cell-like epithelial cells, named seam cells. In C. elegans, the Wnt/β-catenin asymmetry pathway determines the cell fate of most asymmetric divisions (Mizumoto and Sawa, 2007b; i.e., Wnt expressed posterior to the mother cell induces anterior cortical localization of β-catenin in the mother cell, leading to asymmetric cell-fate determination between the two daughter cells). Both ipla-1 mutants and acl-8 acl-9 acl-10 mutants exhibit defects in anterior cortical localization of β-catenin in mother cells and cell-fate determination of the daughter cells. We also found that ipla-1 and acl-10 act cell-autonomously to regulate asymmetric cell divisions of seam cells (Kanamori et al., 2008 and Supplementary Figure 5L, respectively). These data suggest that in ipla-1 mutants and acl-8 acl-9 acl-10 mutants, the altered fatty acid composition of PI in mother cells causes abnormal intracellular distribution of β-catenin, leading to defects in cell-fate determination of the daughter cells.

How is the fatty acid composition of PI involved in asymmetric cell divisions? It has been reported that asymmetric cortical enrichment of a phosphoinositide synthesis enzyme, ppk-1 [PI(4)P5-kinase], is required for asymmetric cell division in the one-celled C. elegans embryo (Panbianco et al., 2008). We also found that seam cell-specific knockdown of ppk-1 results in defective asymmetric division of seam cells (our unpublished data). These data suggest that the cortical asymmetry of phosphoinositides is important for asymmetric cell divisions. Furthermore, a recent study revealed that in HeLa cells, PI(3,4,5)P3 is accumulated in the midsection at the cortex, and the cortical localization of PI(3,4,5)P3 plays a crucial role in the orientation of cell divisions (Toyoshima et al., 2007). These previous reports, together with our findings, suggest that the cortical localization of phosphoinositides in the mother cell is required for correctly determining the fates of the two daughter cells and for the correct orientation of the cell divisions.

It has been assumed that cell-fate determination and orientation of the two daughter cells require the polarized distribution of cortical proteins in the mother cell (Gönçzy, 2008; Siller and Doe, 2009) and that the polarized distribution of cortical proteins is achieved by membrane trafficking (Bilder, 2001; Rodriguez-Boulan et al., 2005). Meanwhile, it has been shown that differences in acyl chains in membrane phospholipids are responsible for the differential lateral distribution and accumulation of lipids in membrane microdomains (i.e., the phospholipids with long and saturated acyl chains preferentially partition into more rigid or highly ordered domains, such as lipid rafts, while those with short or unsaturated tails prefer to enter more fluid regions of the bilayer; Mukherjee and Maxfield, 2004). We speculate that in the mother seam cells of ipla-1 mutants and acl-8 acl-9 acl-10 mutants, the altered fatty acid composition of PI causes abnormal localization of phosphoinositides in the membrane bilayer, leading to the mis-sorting of cortical proteins by retrograde membrane trafficking regulated by tbc-3 and mon-2.

acl-8, acl-9, acl-10 Subfamily Genes Are Evolutionarily Conserved from C. elegans to Mammals

In this study, we showed that acl-8 acl-9 acl-10 mutants have defects in asymmetric division of stem cell-like epithelial cells. acl-8, -9, and -10 form a subfamily of the C. elegans acl/AGPAT family and are the closest homologues of LYCAT/ALCAT1, which is conserved in various species including C. elegans, zebrafish, chicken, and human (Supplementary Figure 4). So far, mammalian LYCATs have been reported to possess acyltransferase activity toward the sn-2 position of anionic lysophospholipids including lysoPI in vitro (Cao et al., 2004; 2009; Agarwal et al., 2006; Zhao et al., 2009). We revealed that mouse LYCAT transfers stearic acid to the sn-1 position of PI as well as the sn-2 position of PI at comparable levels (Supplementary Figure 12). Furthermore, we found that expression of mouse LYCAT rescues the defects in asymmetric division in acl-8 acl-9 acl-10 mutants (Supplementary Figure 5M), indicating that mouse LYCAT is a functional homologue of acl-8, -9, and -10. Interestingly, mouse LYCAT was reported to be highly expressed in hematopoietic stem cells (Wang et al., 2007), which undergo asymmetric divisions to renew themselves and produce the various progeny cells of the distinct blood lineages (Congdon and Reya, 2008; Giebel, 2008). In addition, morpholino-mediated knockdown of zebrafish LYCAT results in a reduction of blood cells (Xiong et al., 2008). Further studies are
expected to reveal an evolutionarily conserved role of the sr-1 fatty acid of PI in the asymmetric division of stem cells.

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